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14. ABSTRACT The main goal of this study is to overcome docetaxel (DTX) resistance in prostate cancer cells by combined delivery of eIF4E siRNA and DTX using dendrimer as a nanocarrier. To this end the objective of this study is to prepare, characterize and test the ability of the combination delivery approach in-vitro in DTX sensitive and resistant prostate cancer cells. We have prepared and characterized the multifunctional delivery system by conjugating DTX to dendrimer and complexing eIF4E siRNA to the resulting conjugate. The DTX-dendrimer conjugate formed complex with siRNA at 20:1 ratio. The dendrimer-siRNA complex was taken up by the prostate cancer cells while the free siRNA was not taken up by prostate cancer cells. The siRNA-dendrimer complex showed gene silencing effect in drug sensitive prostate cancer cells. The IC ₅₀ of DTX in sensitive prostate cancer cells was found to be 50nm. The pre-treatment of the cells with siRNA followed by DTX treatment slightly enhanced cell death of prostate cancer cells. Our future studies will focus on developing DTX resistant prostate cancer cells and testing the combination therapeutic approach in the resistant cell lines.					
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Introduction

Most prostate cancer (PC) patients progress to advanced castration-refractory prostate cancer (CRPC) within a few years of androgen ablation therapy¹. Although docetaxel (DTX) has shown improved survival in CRPC patients, resistance to treatment severely limits patient survival². The treatment induced overexpression of cell survival and anti-apoptotic proteins leads to drug resistance. In this regard eukaryotic translation initiation factor 4E (eIF4E) plays a critical role in regulating the translation of mRNAs that encode several proteins involved in cell growth and survival³. Our goal is to overcome this drug resistance by combined delivery of eIF4E siRNA and DTX using dendrimer as a nanocarrier. To this end the specific aims of this study are i) to test whether a combination of eIF4E siRNA and DTX results in increased activity in PC cells, ii) to prepare and characterize dendrimer-DTX-siRNA nanotherapeutic system, iii) to test the efficacy of nanotherapeutic system in PC3 cells.

Body

Specific Aim 1: To Test the activity of eIF4E siRNA and DTX in PC cells

1a. Combined treatment of eIF4E siRNA and DTX in PC3 cells.

As a first step, the IC₅₀ value of DTX was determined in drug sensitive PC3 cells. The cells were seeded in a 96 well plate at cell density of 10000 cells/well and cultured overnight. The following day, cells were treated with serial dilutions of docetaxel (0.1nM, 0.5nM, 1nM, 5nM, 10nM, 25nM, 50nM, 100nM, and 200nM) for 48 hrs. At the end of treatment, the drug solution was removed and the cell viability was tested by MTT assay. The IC₅₀ of docetaxel in PC3 cells was found to be 50nM (Figure 1)

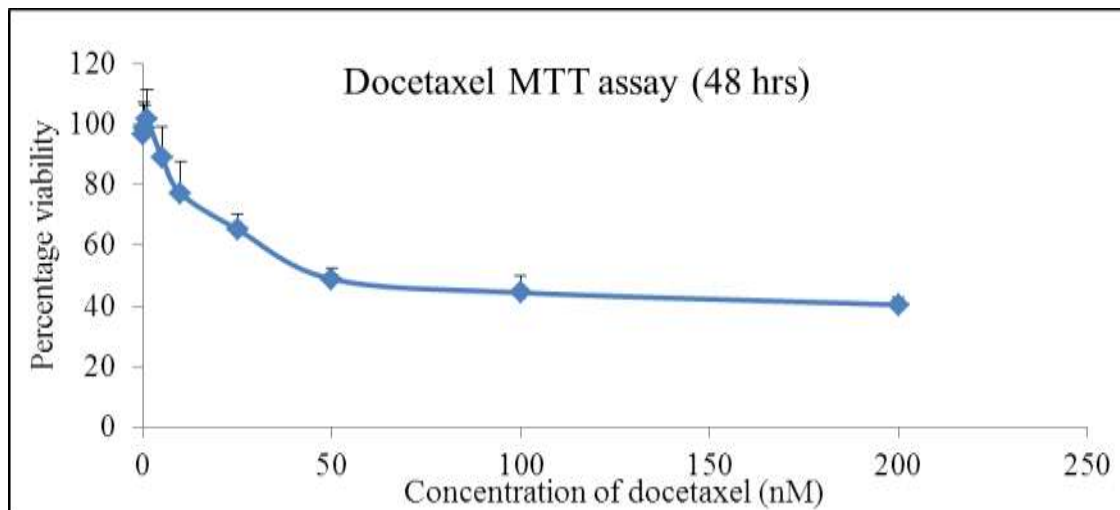


Figure 1. The values are presented as Mean \pm SD, n=3.

To investigate the effects of siRNA on PC3 cells, two separate sets of experiments were performed. In one case, the cells were co-treated with siRNA and docetaxel for 48 hrs. In the second case, cells were pretreated with siRNA (48hrs), followed by treatment with docetaxel (48 hrs). At the end of the treatment, the cell viability was measured using MTT assay. The concentration of siRNA used was 150nM and was complexed with Hiperfect transfection agent (Qiagen, Valencia, CA). Four concentrations of docetaxel were used (1-50nM). As can be seen from Figure 2, the combined application of siRNA and DTX caused slightly higher cell death than either of them alone. However the effect was not significant. This can be attributed to the fact that the drug sensitive PC3 cells do not overexpress eIF4E unlike the DTX resistant PC3 cells.

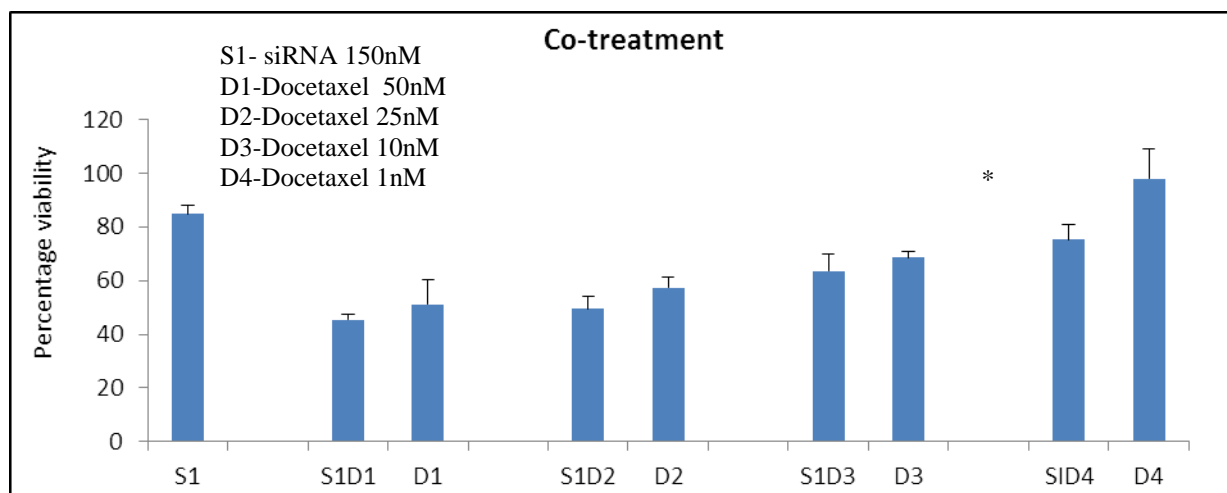


Figure 2. PC3 cells were co-treated with docetaxel and siRNA for 48hrs. At the end, cell viability was measured using MTT assay. The values are presented as Mean \pm SD, n=4. * represents that the values are significantly different ($p < 0.05$) compared to S1 and D4.

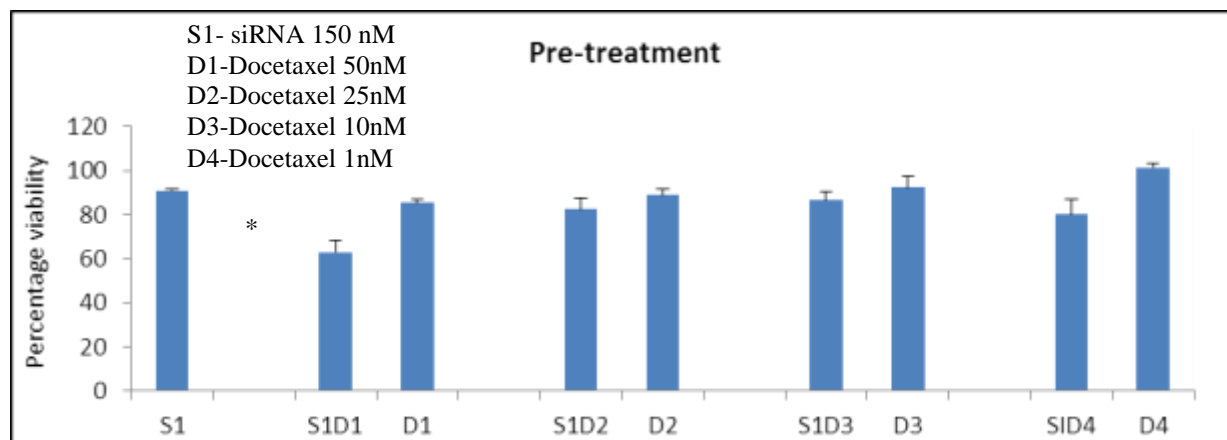


Figure 3. PC3 cells were pretreated with siRNA for 48hrs followed by treatment with docetaxel for 48hrs. At the end, cell viability was measured using MTT assay. The values are presented as Mean \pm SD, n=4. * represents that the values are significantly different ($p < 0.05$) compared to S1 and D1.

Conclusion: Both the experiments showed some slight benefit of using combination treatment (eIF4E siRNA and docetaxel) compared to siRNA or docetaxel alone. These preliminary experiments suggest the potential of combined delivery of docetaxel and siRNA for prostate cancer treatment. The effect of combined delivery is expected to be higher in case of docetaxel resistant PC3 cells which has enhanced levels of eIF4E protein.

1b. Developing DTX resistant PC3 cell line

To develop DTX resistant PC3 cell lines, the cells were treated with three different concentrations of DTX (1-10nM) for about 4-5 months. Cells were treated with DTX for 4-5 days ("on" cycle) and after that the cells were grown in fresh culture media for 4-7 days ("off" cycles). This treatment protocol was followed up to 4-5 months⁴. After 2 months of growth the MTT assay was performed for 5 and 10 nM DTX resistant PC3 cell lines. However there was no significant change in the IC₅₀ values of DTX after 2 months (Figure 4 and 5).

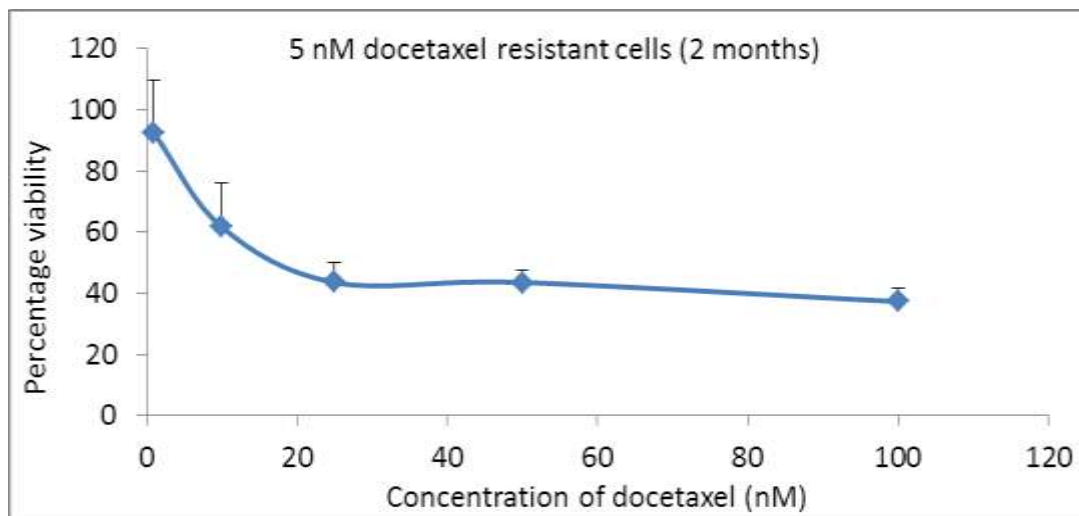


Figure 5. Cell viability of PC3 cells (48 hours) with increasing concentrations of DTX. The PC 3 cells were pretreated with 5 nM of DTX for 2 months before carrying out the MTT assay. The values are presented as Mean \pm SD, n=3.

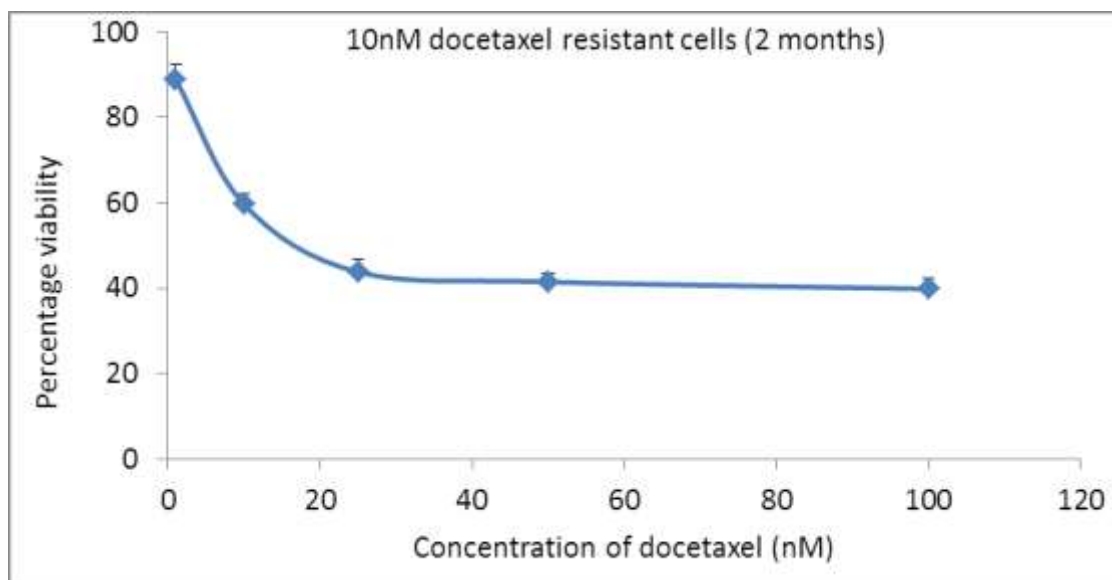
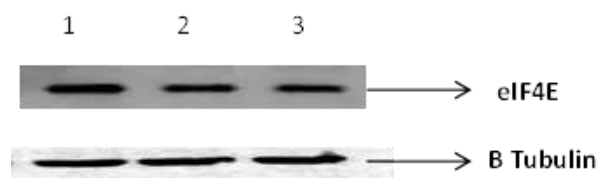


Figure 5. Cell viability of PC3 cells (48 hours) with increasing concentration of DTX. The PC 3 cells were pretreated with 10 nM of DTX for 2 months before carrying out the MTT assay. The values are presented as Mean \pm SD, n=3.

After 2 and 4 month growth of DTX resistant PC3 cell lines the protein was extracted and Western blot was performed. The eIF4E protein levels were measured using eIF4E antibody (Santa Cruz Biotechnology, Santa Cruz, CA). β -tubulin was used as the positive control (Figure 6). There was no significant difference in the expression of eIF4E levels.

a) After 2 months



1st lane: Control PC3 cells

2nd lane: 5nM docetaxel resistant cells
 3rd lane: 10nM docetaxel resistant cells

b) After 4 months

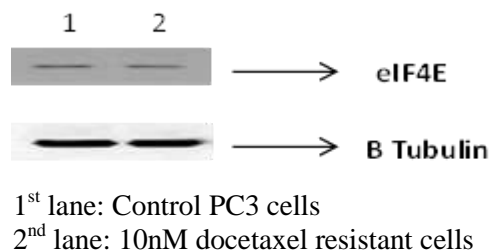


Figure 6. eIF4E bands after 2 (a) and 4 months (b) of DTX (10nm) treatment of PC3 cells.

After 4 months of treatment the PC3 cells did not show any overexpression of eIF4E levels and did not show resistance to DTX treatment. Based on our discussion with other investigators in the field, we understand that the development of resistant cell lines can be quite challenging. After several efforts, we have now procured DTX resistant PC3 cells from a leading Prostate cancer research group in Canada. We are in the process of growing the cells and are hopeful of generating the resistant cell lines during the extension period.

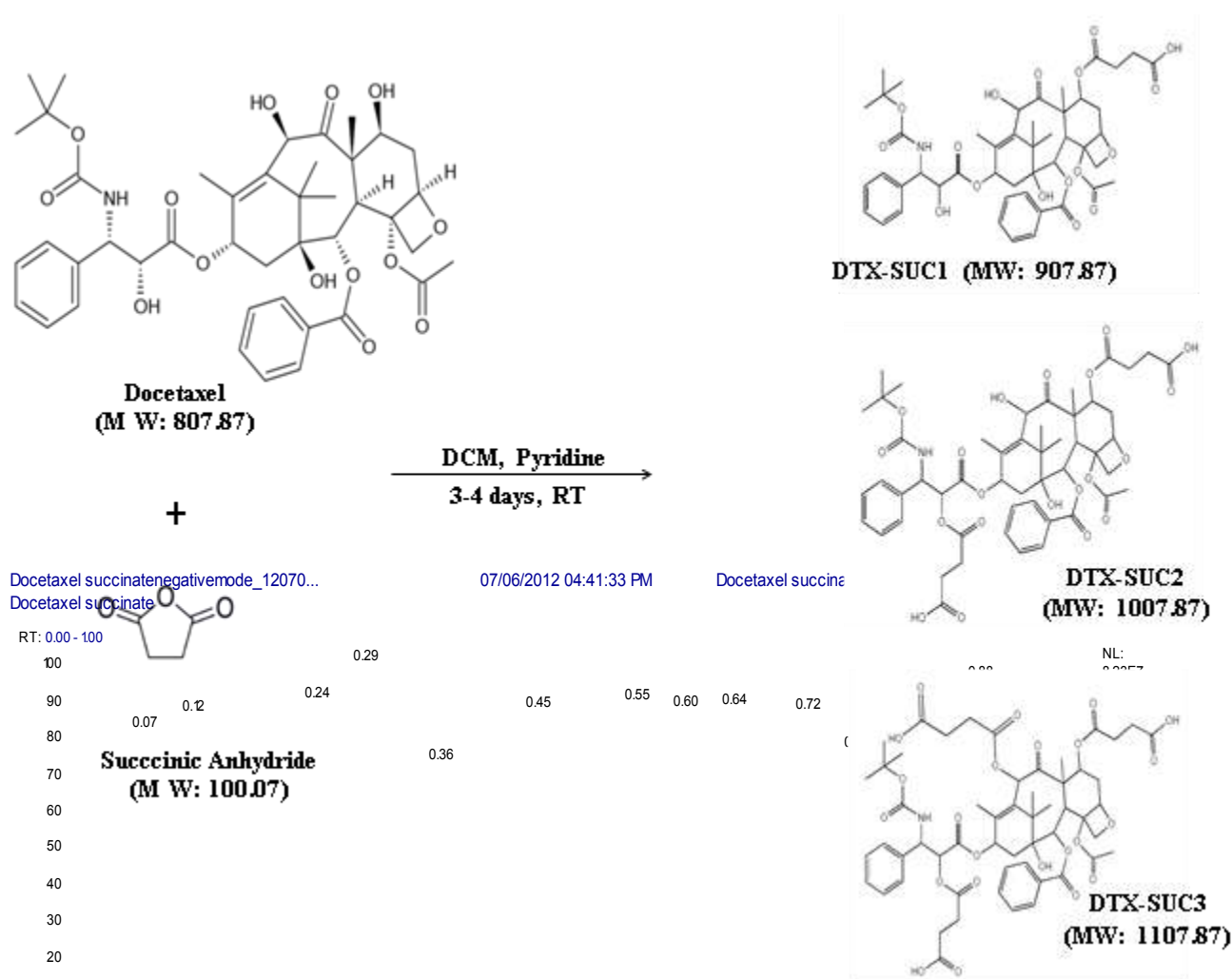
1c. Combined treatment of eIF4E siRNA and DTX in PC3dR cells.

Due to the difficulties in developing the DTX resistant prostate cancer cells, we have not carried out these studies.

Specific Aim 2: Prepare and characterize dendrimer-DTX-siRNA conjugate

2a. Preparation of nanotherapeutic system

As a first step in developing the multifunctional nanotherapeutic system we synthesized the DTX-dendrimer conjugate. DTX was covalently conjugated to 4th generation polyamidoamine dendrimer using hemi-succinate derivative of DTX (DTX-Suc) as the intermediate. The prepared conjugate was characterized using UV-Visible spectroscopy, FT-IR spectroscopy and MALDI TOF to confirm the conjugation. For the preparation of DTX-Suc conjugate (Scheme 1), 500 mg (0.619 mmol) of DTX was dissolved in 6 mL of dichloromethane and to this solution 928 mg (0.928 mmol) of succinic anhydride was added with stirring^{5, 6}. After 5-10 minutes of stirring 500 μ L of pyridine (10 eq) was added to the above mixture. The mixture was kept stirred at room temperature for 3 days. After 3 days the mixture was analyzed using thin layer chromatography (90:10 ethyl acetate/hexane). ESI Mass spectroscopy of the reaction mixture was carried out with methanolic solution of sample introduced by direct infusion using a syringe pump in ion trapped based mass spectrometer (Finnigan LC Q Deca LCMS; GenTech Scientific Ltd.). The mass spectra (Fig. 7) showed that the mixture contains all the possible three succinate derivatives of DTX. To obtain the hemi-succinate derivative of DTX we separated and purified the mixture using the same combination of the solvents through dual channel automated flash chromatography system (W-Prep 2XY; Yamazen Sci. Inc. at 254 nm). After separation through flash chromatography the product was dried using rotary vacuum evaporator (Buchi Rotavapor RII) to get DTX-Suc. DTX-Suc sample was again assessed for the mass using ESI mass spectrometer in similar way as described above. The mass spectra of the separated large fraction (Fig. 8) clearly displayed the peak at 930 (sodium salt derivative of DTX succinate) confirming the synthesis of the intermediate for the final conjugate. DTX-Suc was further characterized by the proton NMR (Fig. 9) and FTIR (Fig. 10) to confirm the synthesis. ¹HNMR was carried out using dimethyl sulphoxide (NMR grade) as solvent. While the FT IR analysis of solid sample was carried out using Thermo Nicolet® FTIR-ATR 380 model (Fig. 10). Both proton NMR and IR spectra confirmed formation of DTX-Suc.



Scheme 1: Synthetic scheme of DTX Succinate. DTX-SUC 1,2 and 3 are the possible DTX succinate derivatives of this reaction which were separated to get DTX-Hemisuccinate derivatives

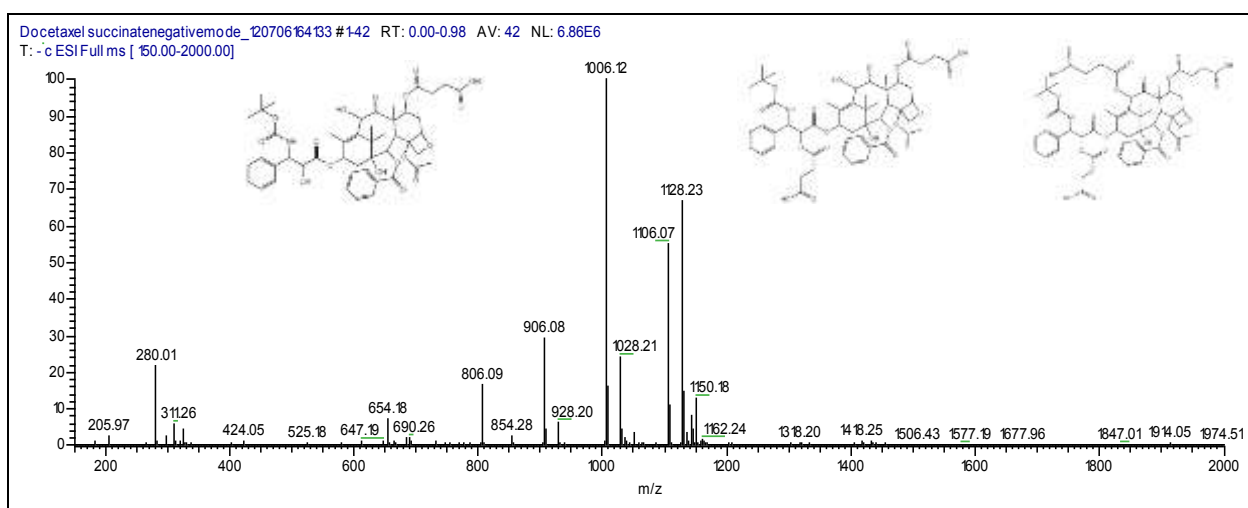


Figure 7: ESI Mass spectra of DTX-Succinate (DTX-SUC1, 2 and 3). (Negative mode)

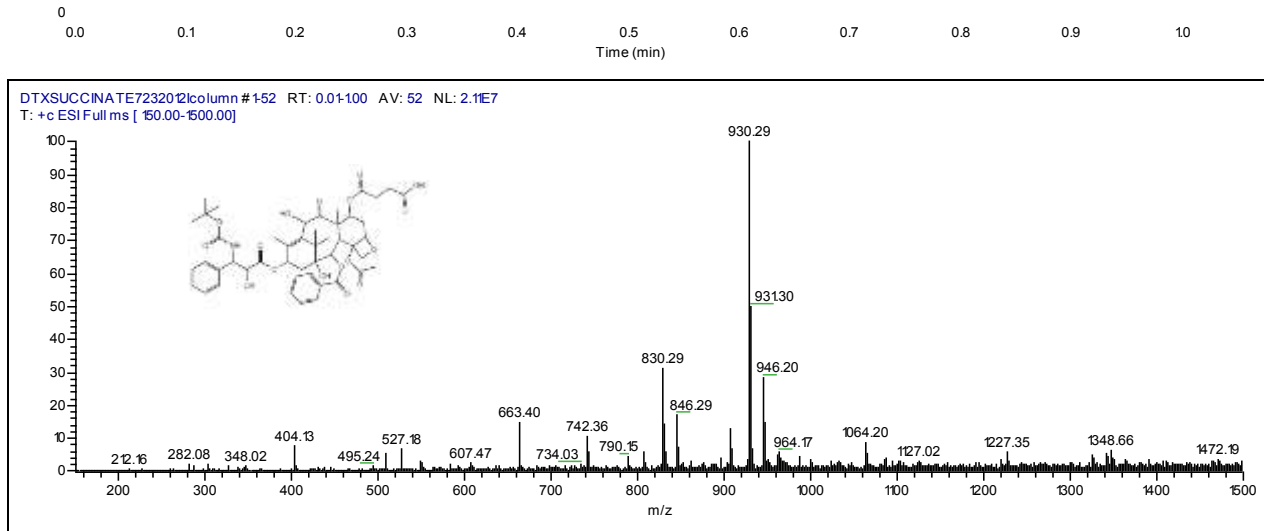


Figure 8: ESI Mass spectra of purified DTX-hemisuccinate (MW 907; negative mode)

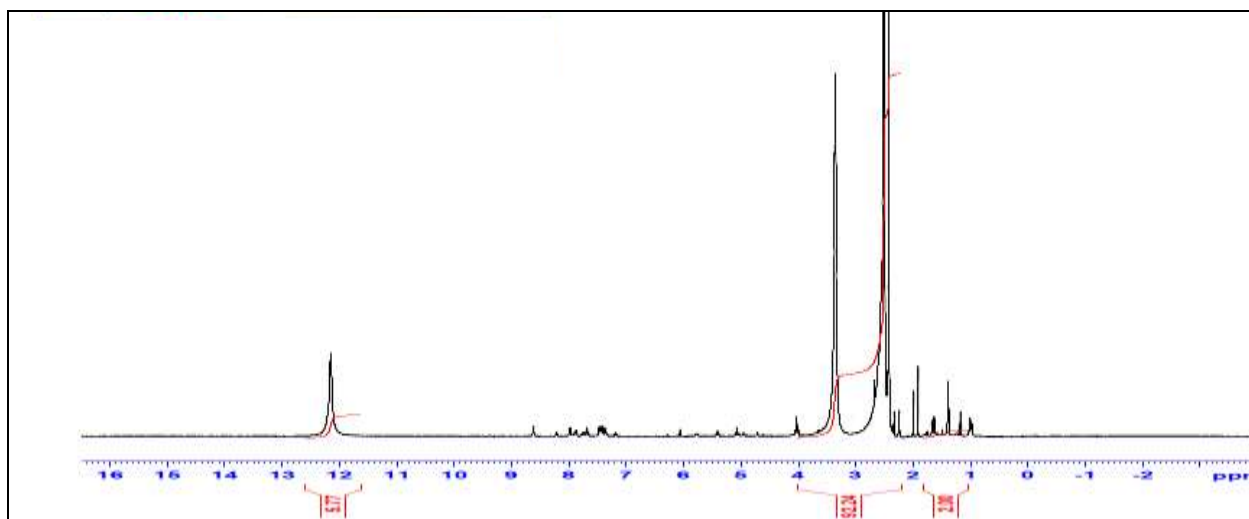


Figure 9: ^1H NMR spectra for docetaxel succinate (DTX-Suc).

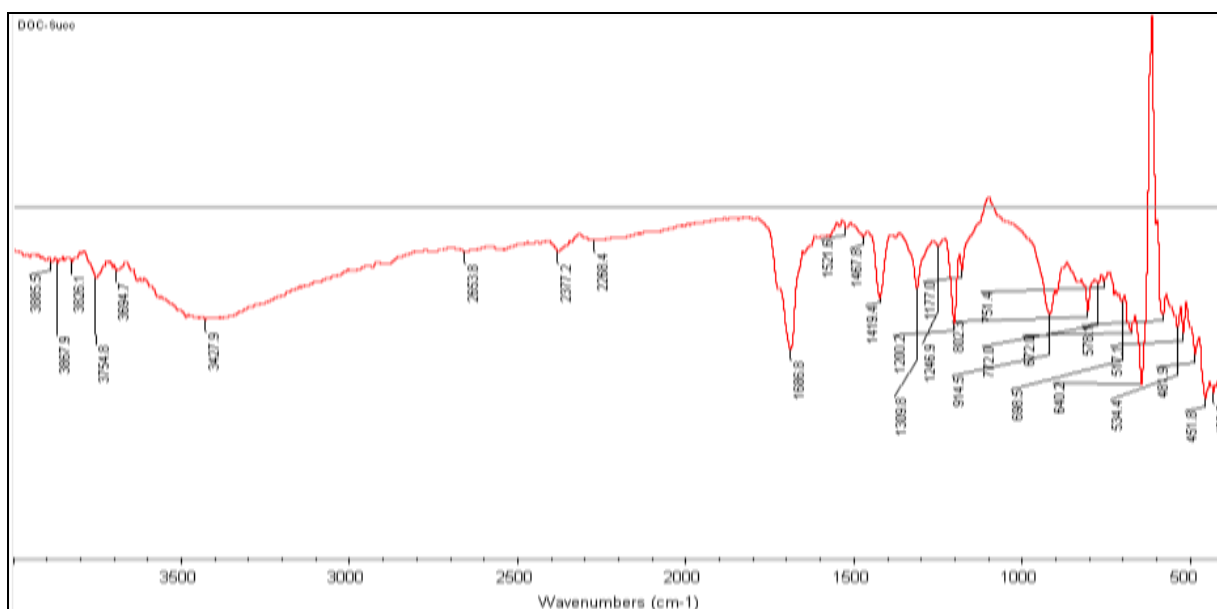
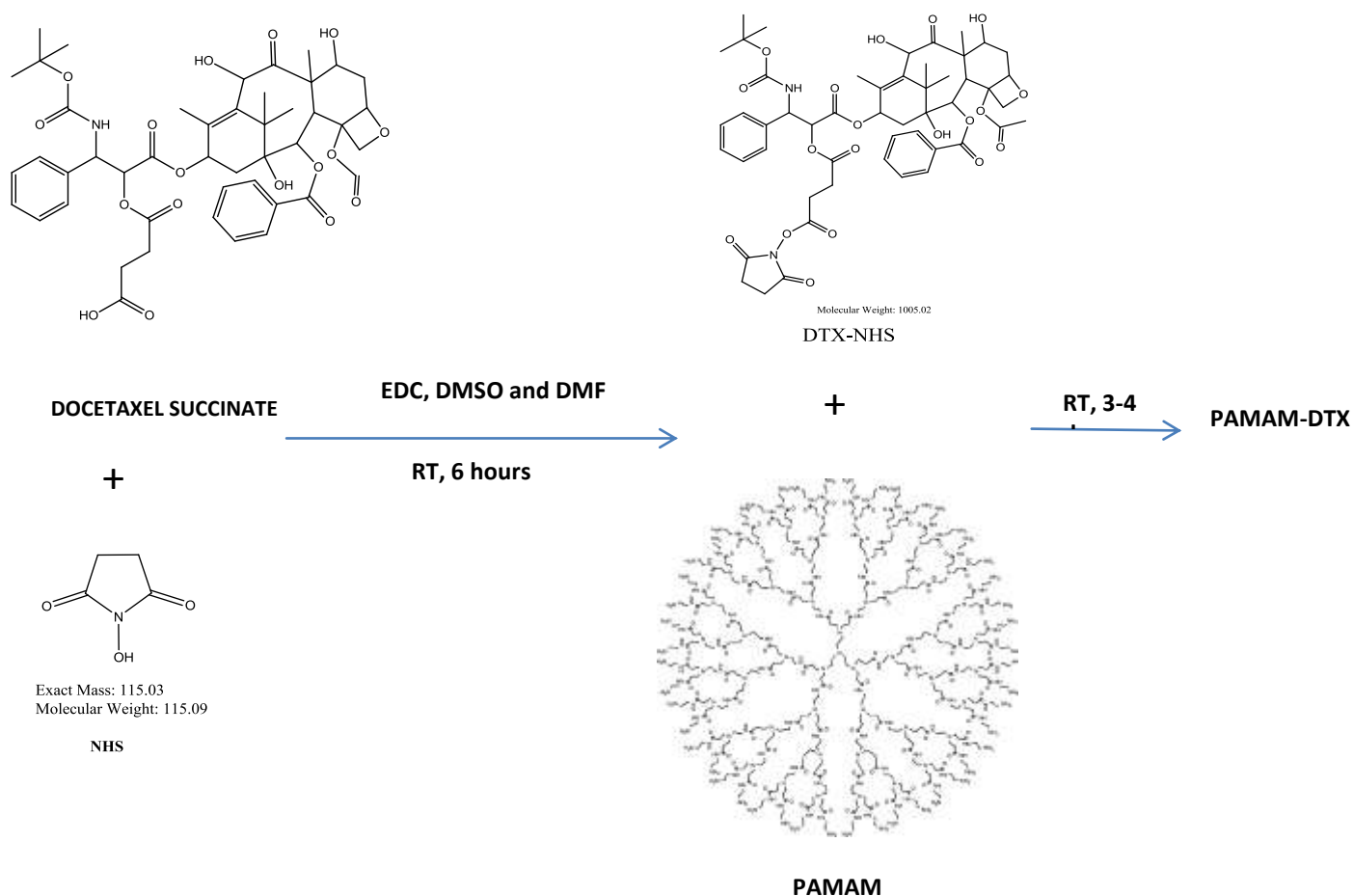


Figure 10: FT-IR spectra for docetaxel succinate (DTX-Suc).

In the second step, we conjugated DTX-Suc to generation 4 polyamidoamine (PAMAM) dendrimer as shown in Scheme 2. Briefly, 140 mg of DTX-Suc was added with 88.55 mg (5 eq) of N-hydroxy Succinimide (NHS) and

then 9 mL of DMF and 3 mL of DMSO was added. To this reaction mixture 35.42 mg (1.2 eq) of EDC was added in inert environment of nitrogen. The reaction mixture was stirred for 4-6 hours.



Scheme 2: Schematic synthesis steps of conjugation of PAMAM dendrimers to DTX-Suc.

After 4-6 hours of stirring 100 mg of PAMAM dendrimer was added and the reaction was carried out with constant stirring and room temperature for 3 days⁷. The mixture was extracted using ethyl acetate and deionized water. The aqueous extract was dialyzed against 20:80 mixture of DMSO and water for 24 hours using dialysis membrane cassette (MWCO 10000 Da). After 24 hours the aqueous part was again dialyzed against fresh deionized water two times for 12 hours. After dialysis the whole content was lyophilized using Freeze dryer (VirTis). The conjugate was characterized using UV visible spectroscopy, FT-IR spectroscopy and proton NMR. The results of the UV –Visible spectroscopy displayed a shift of 10 nm (Figure 11) i. e. absorbance maxima in case of PAMAM dendrimer was 280 nm (Figure 11A) while for the conjugate it was 290 nm (Figure 11B). The conjugate was further characterized by FTIR (Figure 12) spectroscopy. The IR spectra supported the conjugation as there were strong peaks around 1650-1700 cm^{-1} clearly indicating the formation of amide bond. Additionally peak was also obtained between 550 to 600 cm^{-1} indicating, the presence of aromatic residue (Figure 12). Proton NMR was carried out using methanol as solvent. The NMR spectra further confirmed the synthesis of PAMAM–DTX conjugate with chemical shift at δ 6.5, indicating the presence of aromatic residue in the compound (Figure 13).

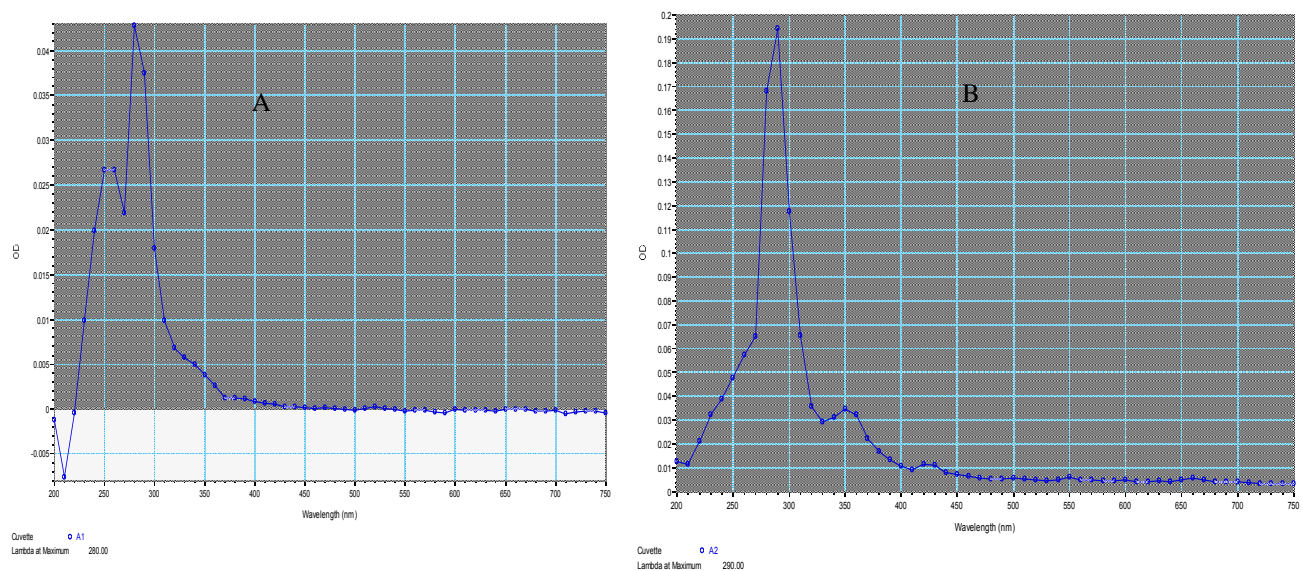


Figure 11: UV-Visible spectra of (A) PAMAM Dendrimers; (B) PAMAM-DTX conjugate in DMSO.

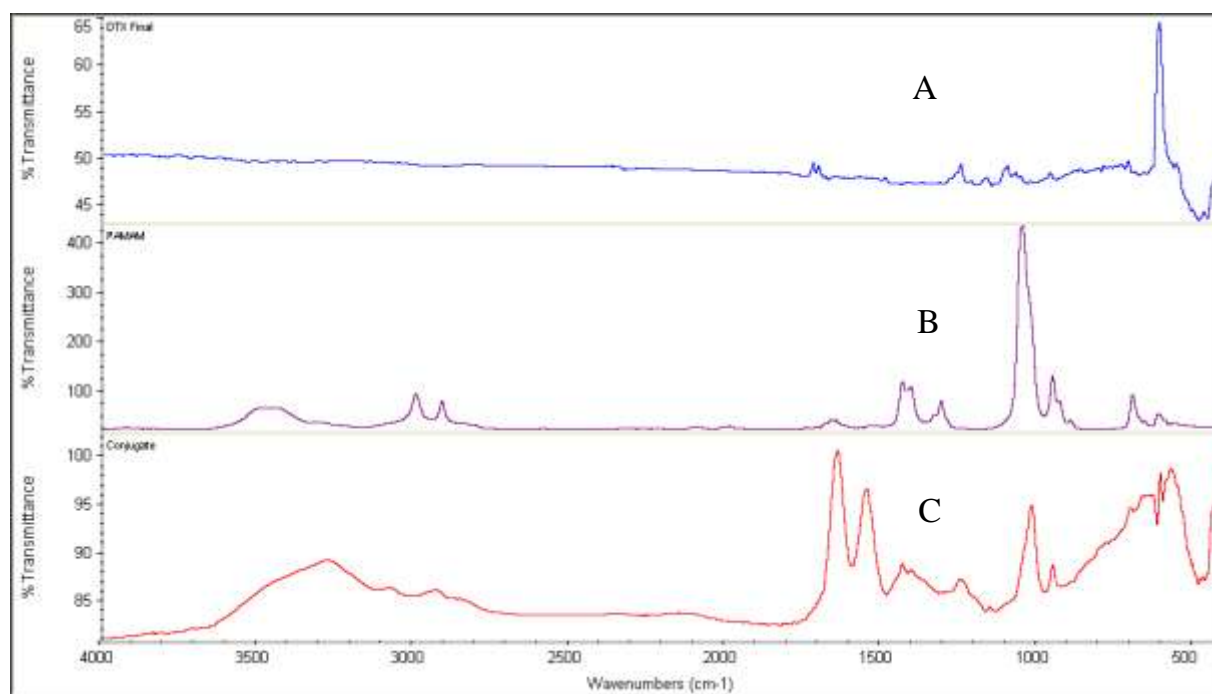


Figure 12: FTIR spectra of (A) Docetaxel; (B) PAMAM 4.0G Dendrimer (C) PAMAM-DTX conjugate.

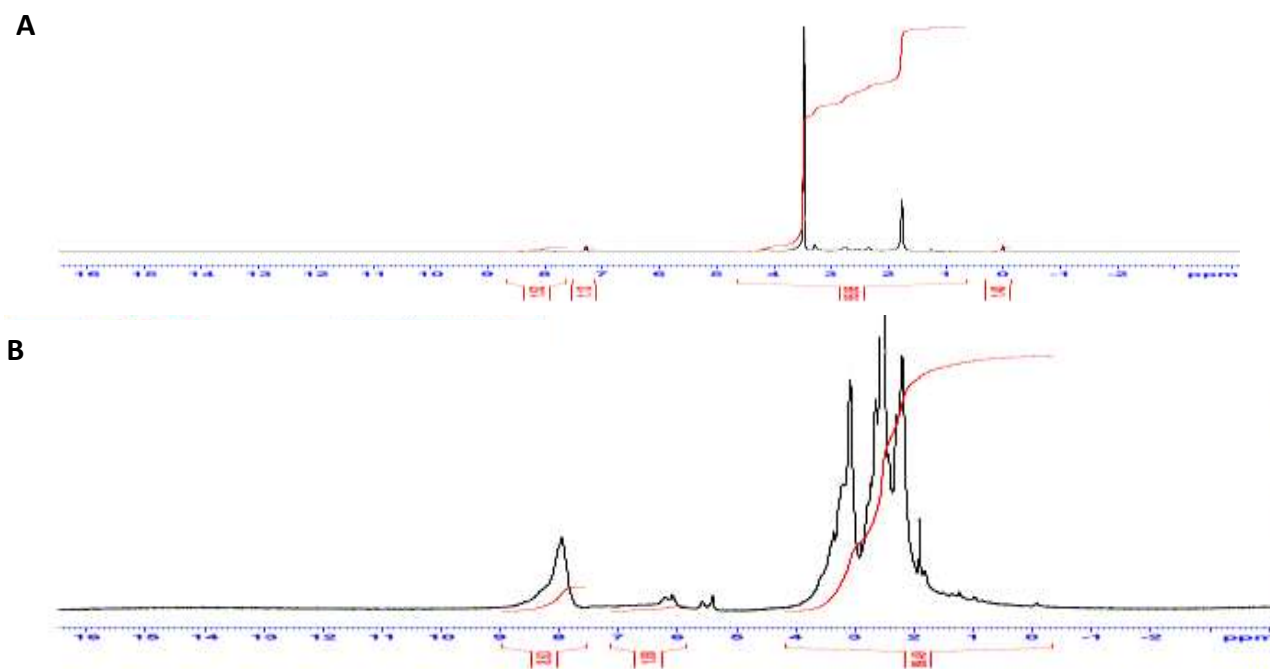


Figure 13: ^1H NMR spectra of PAMAM dendrimer (A); PAMAM-DTX Conjugate (B) (in Methanol).

To characterize the molecular weight and determine the number of DTX molecules in the conjugate, Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (Brucker Daltonics Biflex IV) was used. The mass spectrometer was operated in the positive linear mode using a pulsed nitrogen laser, a sample target control and data analysis software. Three different matrices including 2, 4, 6-trihydroxyacetophenon monohydrate (THAP), DHB (2, 5 dihydroxy benzoic acid) / Fucose (1:1) mixture, α -cyano-b 4 hydroxy cinnamic acid were used to carry out the mass spectrometry^{8, 9}. The mass spectra were processed and averaged from 200 shots per individual selection on a sample-matrix spot Briefly, 10 nmolar dendrimer/dendrimer conjugate solution was prepared in 9/1 (v/v) 0.1% TFA/ACN (Trifluoroacetic acid and Acetonitrile), separately. 10 mg of matrix (α -cyano-4hydroxycinnamic acid or 1:1 DHB/Fucose) was dissolved in 1mL of 1:1 mixture of deionized water and acetonitrile. Sample was prepared by mixing the sample solution (1 μL) with the matrix solution (1 μL) on a stainless steel probe tip, and this mixture was allowed to dry at room temperature. Samples were kept in 0.5, 1 and 2 μL quantities on stainless steel plate. In case of THAP a different method was followed based on reported literature⁸. THAP was dissolved in 1/1 (v/v) 0.1% TFA/ACN mixture (10 mg/mL). Two parts of THAP solution and 1 part sample (0.14 mg/mL or 10 nmolar solution of dendrimer in 9/1 (v/v) 0.1% TFA/ACN) was mixed and 0.5, 1 and 2 μL was used for spotting at stainless steel microtiter. In case of α -cyano-b 4 hydroxy cinnamic acid matrix, DTX PAMAM conjugate displayed the molecular weight 22495.481 [M^+] and 11062.415 [M^{2+}] (Fig. 14a) whereas for PAMAM dendrimer it was 13716 [M^+] and 6740.829 [M^{2+}] (Fig 14b). The theoretical molecular weight of fourth generation dendrimer is 14217 and the MALDI TOF spectrum is close to the theoretical value and is consistent with literature reports^{8, 9}. The molecular weight of the DTX-PAMAM conjugate was 22495.481 which indicate the conjugation of approximately 9 molecules of DTX per molecule of PAMAM dendrimer. In case of THAP and DHB/Fucose matrices did not work and so all further characterization will be done using the α -cyano-b 4 hydroxy cinnamic acid matrix.

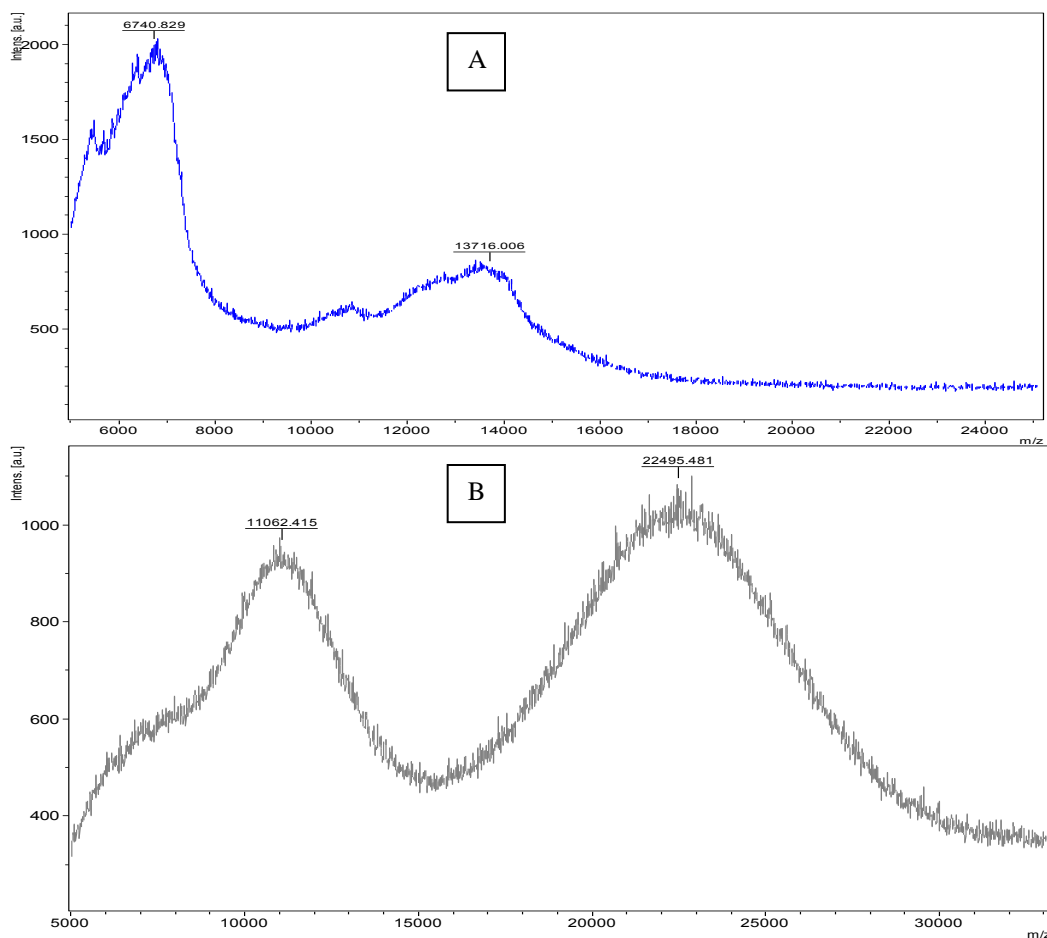


Figure 14: MALDI TOF spectrum of PAMAM (A) and DTX-PAMAM conjugate (B) using α -cyano-b 4 hydroxy cinnamic acid as matrix.

We next developed the dendrimer siRNA complex using different N/P ratio. Briefly, dendrimer and siRNA was incubated at different molar ratios (1:1, 5:1, 10:1) in RNase free water for about 30 minutes. The formation of complex was confirmed using agarose gel electrophoresis. Agarose gel electrophoresis was performed using TBE buffer at 100 mV for 30 minutes. Briefly, 2% agarose was dissolved in 1x TBE buffer with addition of 0.5 $\mu\text{g/mL}$ solution of ethidium bromide. Each well was loaded with 10 μL of the dendrimer-siRNA complex (with different N/P ratio) mixed with loading dye, such that the minimum quantity of siRNA in each well was 1 μg (Figure 15). Photograph was taken using BIORAD, Molecular Imager Chemi Doc XRS after 30 minutes. The results showed that the dendrimer-siRNA complex was formed at 1:1 ratio

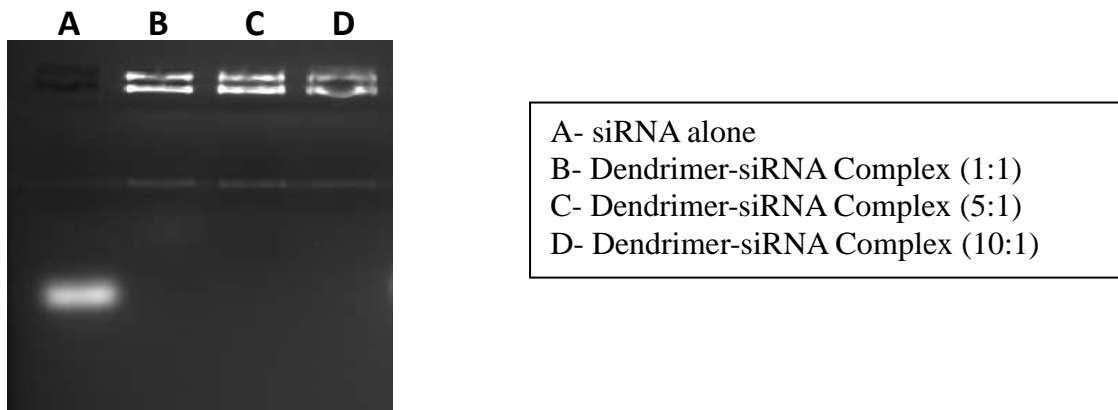


Figure 15: Agarose gel electrophoresis to confirm the complex formation between dendrimer/dendrimer-DTX conjugate and siRNA at various Nitrogen/Phosphorus (N/P) ratios (BIORAD, Molecular Imager Chemi Doc XRS).

Similarly, the complex was also developed for dendrimer-DTX and siRNA following similar procedure as mentioned above. However N/P ration chosen in this case was 1:1, 5:1, 10:1 and 20:1 (Figure 16).

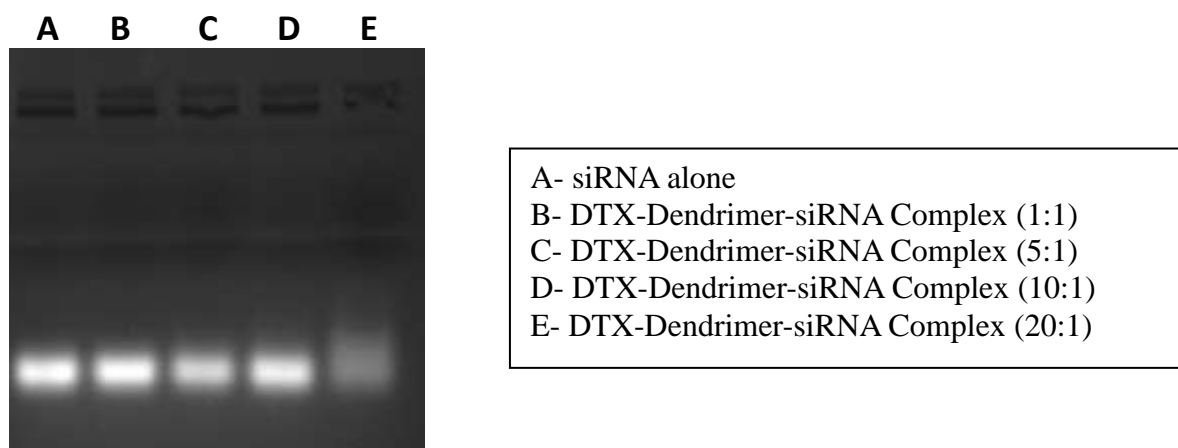


Figure 16: Agarose gel electrophoresis to confirm the complex formation between dendrimer-DTX conjugate and siRNA at various Nitrogen/Phosphorus (N/P) ratios (BIORAD, Molecular Imager Chemi Doc XRS).

The results show that the conjugation of DTX influenced the complex formation between dendrimer and the siRNA and that a higher dendrimer-DTX:siRNA ratio was required for complex formation. Further studies will focus on optimizing the dendrimer-DTX:siRNA ratio by optimizing the number of DTX molecules and varying the dendrimer generation.

2b. Release studies

The release of DTX and siRNA from the nanotherapeutic system is currently in progress.

Specific Aim 3: Test the activity of nanotherapeutic system in PC cells

3a. Activity in DTX sensitive PC3 cells

As a first step we tested the cell uptake of siRNA-dendrimer complex using fluorescence microscopy. PC3 cells were seeded in 6 well plates at cell density of 3×10^6 cells/well and cultured overnight. The following day culture media was removed and cells were washed 2-3 times with PBS. The cells were then treated with dendrimer-siRNA (TAMRA labeled) complex (10:1), TAMRA labeled siRNA alone, separately, in serum free culture media, for 2 and 6 hrs. After 2 hours media was removed from each well and cells were again washed three times with PBS. The cell uptake was observed under inverted fluorescence microscope (20X; Zeiss Axio Observer). Same procedure was followed for the cell uptake observations at 2hrs hours. As seen from Fig. 17, cell uptake of siRNA-dendrimer complex was observed while the free siRNA was not taken up by the cells.

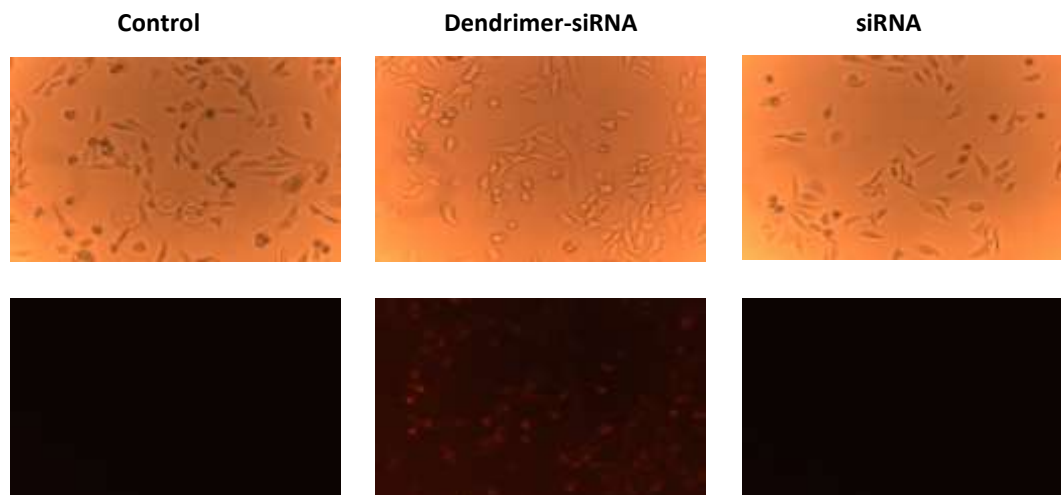
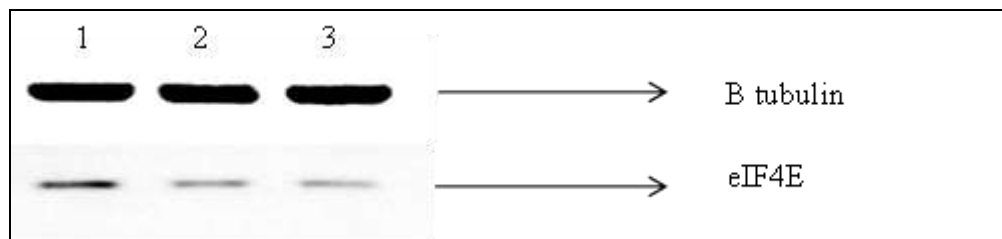


Figure 17: Cell Uptake studies (2 hrs) performed using TAMRA labeled siRNA in PC3 cells (20X; Inverted fluorescence microscope, Zeiss Axio Observer).

To test the gene silencing effect PC3 cells were treated with eIF4E siRNA complexed with the Hiperfect transfection agent (Qiagen, Valencia, CA) for 3-4 days. At the end of treatment, the protein was extracted and western blotting was performed. The eIF4E protein levels were measured using eIF4E antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Beta tubulin was used as the positive control. The eIF4E levels decreased with increase in treatment time demonstrating the ability of the dendrimer-siRNA complex to silence the eIF4E expression (Fig 18).

a)



1st lane: Control PC3 cells

2nd lane: PC3 cells treated with eIF4E siRNA (150nM) for 3 days

3rd lane: PC3 cells treated with eIF4E siRNA (150nM) for 4 days

b)

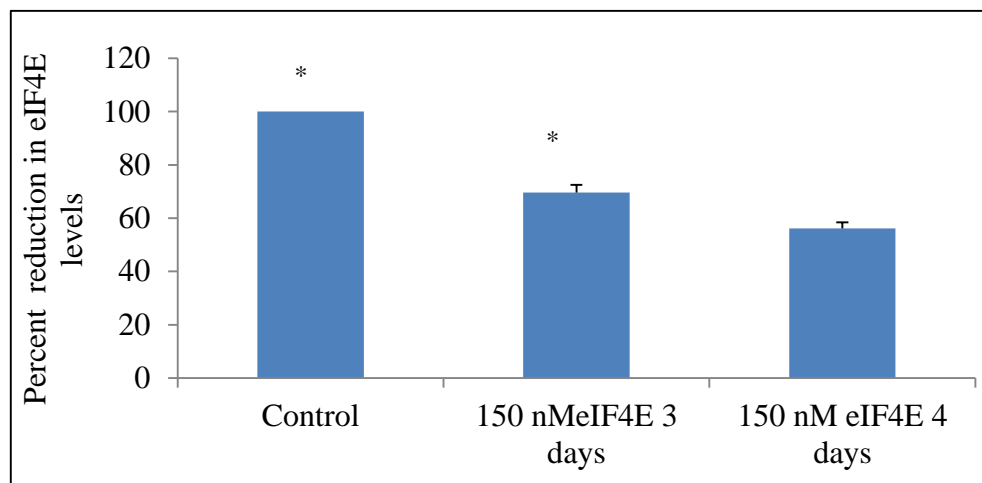


Figure 18. a) eIF4E protein bands and the intensity of the bands is represented as a function of treatment time (b) in PC3 cells. The values are presented as Mean \pm SD, n=3.

3b. Activity in DTX resistant PC3 cells

These studies are yet to be carried out and will be studied once the DTX resistant cell lines are developed.

Key Research Accomplishments

- A synthesis method was developed for DTX- dendrimer conjugate
- Complex formation of siRNA with dendrimer enhanced the cell uptake of siRNA in PC3 cells
- Treatment of PC3 cells with eIF4E siRNA reduced the levels of eIF4E levels.

Reportable Outcome

- Presented poster entitled “Dendrimer Based Drug Delivery System for Prostate Cancer” at 22nd Annual Pharmacy Research Presentations and Keo Glidden Smith Fall Pharmacy Convocation, October 29, 2012 at College of Pharmacy, South Dakota State University Brookings USA.

Conclusions

So far we have successfully developed a synthetic method to conjugate DTX to dendrimer and also studied the dendrimer:siRNA ratio for complex formation. The cell uptake of siRNA-dendrimer complex was demonstrated. Further the ability of the siRNA to suppress the levels of eIF4E levels was demonstrated in prostate cancer cells. So far we have not been able to develop DTX resistant prostate cancer cells but have procured the DTX resistant cells from a different group. Our future studies will focus on growing the DTX resistant cells and confirming the overexpression of eIF4E. Further our studies will focus on optimizing the number of DTX and siRNA molecules in the nanotherapeutic system, testing the in-vitro release as well as testing its activity in DTX resistant prostate cancer cells. The knowledge gained so far demonstrates the feasibility of developing a multifunctional nanotherapeutic system and the results can be extended to conjugate other anti-cancer drugs and siRNA for prostate cancer and other cancers.

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